A Study of Neuron Specific Cationic Amino Acid Transporter rCAT3

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CONTENTS

GENERAL INTRODUCTION

In mammalian central nervous system, a large number of neurons exist. They mutually send and receive messages, and this function is performed by various proteins encoded on its genome. It has been estimated that 20,000 genes are specifically expressed in the mammalian nervous system. The neuronal interactions and pathways are phenomenally complex, ranging from brain development to daily central nervous system functions such as psychiatric states and behavior. To study the specific protein produced by expression of each gene can lead us to understand the basis of behavior, awareness, and consciousness.

One of the neuron-to-neuron signals is nitric oxide, synthesized from intracellular L-arginine by nitric oxide synthase. Recently, L-arginine incorporation has been focused, because it may be critical for the production of nitric oxide. In mammalian brain, the activity of L-arginine incorporation was confirmed, but the protein with the activity has not been clarified. In this study, I focused on the gene encoding neuronal specific L-arginine transporter in mammalian brain, and succeeded in molecular cloning of the gene (named rCAT3). In part I, I describe the study of "molecular cloning and characterization of rCAT3." In Part II, I describe the study of "physiological roles of rCAT3".

SUMMARY

A cDNA clone rCAT3 (rat cationic amino acid transporter 3) which encodes a novel member of the murine CAT family was isolated. The protein encoded by rCAT3 contained 619 amino acids of which 53 - 58 % were identical with those of the murine CAT family proteins previously described (mouse CAT1, CAT2a, CAT2b and rat CAT1). Transient expression of rCAT3 and L-[^{14}C]arginine incorporation experiments in COS7 cells verified a high affinity system y$^+$ transporter activity of rCAT3. First, rCAT3-mediated L-[^{14}C]arginine incorporation was time-dependent and saturable with half-saturation constant (Km) values of 103 ± 12 µM (means $\pm$ SEM, n=3). Second, the incorporation was specific for cationic amino acids as evidenced by the inhibition by L-arginine, L-lysine and L-ornithine. Third, neither sodium nor chloride ions in the extracellular medium were required for the activity. And fourth, the incorporation was inhibited by high potassium-induced membrane depolarization. On northern blot using RNAs from various rat tissues, the expression of rCAT3 mRNA was restricted to brain. These results indicated a role of rCAT3 in the system y$^+$ transporter activity in the nervous tissue.

INTRODUCTION

Nitric oxide (NO)[1], a highly diffusible molecule involved in signal transduction in brain, is formed from the terminal guanidino group of L-arginine by an enzyme NO synthase (NOS) (I - 1). Of the two classes (constitutive and inducible) of NOS isoforms, neuronal NOS belongs to the constitutive type and it is generally accepted that calcium/calmodulin signaling is the primary mechanism to regulate its activity (I - 1,2). Several lines of evidence suggested that, besides the regulation of NOS activity, NO synthesis is also regulated by availability of the substrate L-arginine (I - 3) and that L-arginine is provided to NOS via transport from the extracellular space rather than from the intracellular metabolic pool. For example, N-methyl-D-aspartate-induced NO production in neuroblastoma cells depended on extracellular L-arginine (I - 4). NO synthesis by lipopolysaccharide-activated macrophage was also dependent on extracellular L-arginine and was inhibited by L-lysine or L-ornithine (I - 5). Inhibitory effects of these cationic amino acids on NO synthesis were also observed in brain synaptosomes (I - 6). Furthermore, exogenous L-arginine enhanced NO synthesis in brain slices (I - 7,8).

The amino acids are incorporated from exterior medium to intracellular space via carrier proteins on the cell surface. Among the several types of amino acid transport systems distinguished by substrate specificity, cationic amino acids such as L-arginine, L-lysine and L-ornithine share the same transporter with a sodium-independent activity, called system y^+ (I - 9). Although the presence of a system y^+ activity in neuronal/glial cells has been demonstrated by functional studies (I - 10,11), molecular basis for the activity remains unclear.

At least two genes, mCAT1 (mouse cationic amino acid transporter 1) and mCAT2, have been identified and verified to encode a high affinity (Km = ~100 mM) system y^+ activity in murine, non-neuronal tissues. mCAT1,

originally identified as a receptor for an ecotropic retrovirus (I - 12), was later found to encode a system y^+ activity by expression studies in *Xenopus* oocytes (I - 13,14). mCAT1 is widely expressed in various tissues and cell lines and believed to be a ubiquitous form of the CAT gene family (I - 14). mCAT2 gives rise to two alternative splice forms, mCAT2a and b. mCAT2b, originally identified as a T-cell early activated gene (I - 15), was also found to encode a system y^+ activity with properties similar to that encoded by mCAT1 (I - 16). mCAT2b has been shown to be expressed in some types of cells including T cells and vascular smooth muscle cells (I - 17). A comparative study on the distribution of mCAT2b, however, is lacking and it is not known whether neuronal/glial cells express this transporter. mCAT2a does not encode system y^+ but a low affinity, high capacity transporter (I - 18). The expression of mCAT2a is restricted to liver (I - 18).

By RNase protection assay, Stoll et al. (I - 19) demonstrated a marked enrichment of the rat CAT1 (rCAT1) mRNA in brain capillary, suggesting a role of rCAT1 in amino acids transport by endothelial cells at the blood brain barrier. The relatively low expression level of rCAT1 in the whole brain mRNA might imply the presence of a brain-specific, yet unknown transporter distinct from rCAT1. More recently, Wu et al. (I - 20) examined the tissue distribution of rCAT1 mRNA and found a ubiquitous distribution of 7.9 kb hybridization signal and a brain-specific 3.4 kb hybridization signal, the latter of which might represent a brain-specific homologue of rCAT1.

In light of the regulation of NO synthesis by L-arginine transport, I wished to identify the molecular basis for the system y^+ activity in brain. The current study employed homology screening of a rat brain cDNA library and led to the isolation of a novel member of the CAT gene family, designated here as rCAT3.

EXPERIMENTAL PROCEDURES

Probe preparation by reverse transcription-PCR - Total RNA was extracted from rat cerebellum as described (I - 21). cDNA was synthesized from 1 μg of the total RNA by SuperScript II reverse transcriptase (Gibco BRL, Tokyo, Japan) using random hexamers as primers. A part of the reaction mixture was directly used as a template for PCR. The sequences of the primers used for PCR were 5' - GGTCTTACGGTACCAGCCAG - 3' (sense) and 5' - GGACGCTTCCTCACTG - 3' (antisense). These sequences correspond to the nucleotide sequences at 1461-80 and 1977-96 of mCAT1 (I - 12). The PCR contained 1 μM dNTPs, 67 mM Tris-Cl (pH 8.8), 16.7 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 0.5 mM dimethylsulfonate, 2 mM $MgCl_2$, 2.5 units of Taq DNA polymerase (Takara, Tokyo, Japan), 1 μM of each primer and the template. Thirty-five cycles of the following temperature condition were used for amplification; 94 °C for 1 min, 55 °C for 2 min and 72 °C for 2 min, and final extension at 72 °C for 10 min. An amplified DNA was separated on an agarose gel, extracted, subcloned into pCR1000 (Invitrogen, NV Leak, Netherland) and then sequenced using a Bcabest™ dideoxy sequencing kit (Takara). The PCR product was labeled with [^{32}P]dCTP (110 TBq/mmol, Amersham, Buckinghamshire, UK) using a random primers DNA labeling system™ (Gibco BRL) and used as a hybridization probe.

cDNA cloning - A cDNA library from adult Wister rat brain poly (A)$^+$ RNA was constructed in lgt10 vector (Staratagene, La Jolla, CA) (I - 22) and hybridization screening of the library (~10^6 clones) was performed as described (I - 23). A single positive clone (λrCAT3) was extracted from the plaque and the *EcoRI* fragment of the phage was subcloned into pBlueScript SK+ (Stratagene) to give pBSKrCAT3. The nucleotide sequence of pBSKrCAT3 insert was determined as described above.

Transient expression of rCAT3 in COS7 cells and L-[14C]arginine

incorporation assay - The *EcoRI* fragment of lrCAT3 was subcloned into a

mammalian expression vector pME18sf- to give pMErCAT3. COS7 cells were

routinely maintained in Dulbecco's modified Eagle's Medium supplemented

with 10% fetal bovine serum in a CO_2 incubator. The cells, seeded on six-well

plates (10^5 cells per well), were transfected with pMErCAT3 or with vector

plasmid (for basal incorporations) using lipofectamine™ (Gibco BRL)

according to the manufacture's instructions. Two days later, they were

subjected to L-[14C]arginine incorporation assay. The cells were washed twice

with HEPES-buffered saline (HBS) (150 mM NaCl, 10 mM HEPES pH 7.5, 1

mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl) and further incubated in HBS at 37 °C for

10 min. The reaction was started by changing the media to HBS containing L-

[14C]arginine (11 GBq/mmol). The concentrations of L-[14C]arginine applied

and the reaction time were indicated in the figure legends. The reaction was

stopped by washing the cells three times with ice-cold HBS. The cells were

then lysed in 0.2 N NaOH/1% sodium dodecyl sulfate and the radioactivity

incorporated was determined by a solid scintillation counter. Incorporation

assays with L-[3H]lysine (3.55 TBq/mmol) and L-[14C]ornithine (9.69

GBq/mmol) were done in exactly the same way. All of the radioactive amino

acids were obtained from Amersham. Protein concentration of the cell lysate

was determined using a MicroBCA kit (Pearce, Rockford, IL). All of the

unlabeled amino acids and the three NOS inhibitors (N^G-monomethyl-L-

arginine [L-NMMA], L-N^5-(1-iminoethyl)-ornithine [L-NIO], N^G-nitro-L-arginine

methyl ester [L-NAME]) used for the inhibition experiments were obtained from

Sigma (St. Louis, MO).

Northern blotting - The probe was prepared by labeling the *EcoRI* fragment of pBSKrCAT3 with [^{32}P]dCTP (110 TBq/mmol, Amersham) exactly as described above. A rat multiple tissue northern blot membrane was obtained from Clontech (Palo Alto, CA). Blotting procedures were as described (I - 23). The blot was visualized for radioactivity with BAS2000 image analyzer (Fujitsu, Tokyo, Japan).

Statistical analysis - Where necessary, statistical analysis was done by ANOVA (analysis of variance).

RESULTS & DISCUSSION

rCAT3 is a novel member of the murine CAT family - cDNA prepared by reverse transcription from rat cerebellum RNA served as a template for PCR. A ~550 bp product was obtained using a primer set derived from the 12th and 14th TMs of mCAT1 (I - 12). The deduced amino acid sequence of this PCR product displayed ~70 % identity with that of the corresponding region of mCAT1 and served as a probe for library screening. By hybridization screening of a rat brain cDNA library under high stringency conditions, one positive clone (λrCAT3) was isolated.

The nucleotide and deduced amino acid sequences of the λrCAT3 insert (Fig. I - 1A) indicated that it encodes a complete coding sequence of rCAT3. The 5'-proximal ATG triplet is followed by a 1857 bp open reading frame encoding 619 amino acid residues with a calculated molecular weight of ~67 kilodalton. The sequence around this ATG fits the consensus sequence for eukaryotic translation initiation sites (I - 24). rCAT3 lacks a readable signal sequence in the N-terminus (I - 25). Fig 1B depicts a putative membrane topology of rCAT3 predicted by a hydrophobicity analysis (GeneWorksTM, IntelliGenetics, Cambell, CA). rCAT3 contains fourteen putative TMs. One potential N-linked glycosylation site is located on an extracellular loop between the 5th and 6th TMs and one potential protein kinase C (PKC) phosphorylation site is located on an intracellular loop between the 10th and 11th TMs. Another structural feature of rCAT3 is a leucine zipper consensus sequence in the first TM (Fig. I - 1A). Originally identified as a functional unit required for dimerization of DNA-binding proteins (I - 26), the leucine zipper motif has also been found in membrane proteins including voltage gated potassium channels (I - 27), glucose transporters (I - 28) and some neurotransmitter transporters (I - 29), where it may mediate subunit oligomerization.

A

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                                                          CTCTTCAATTCCAACTAGA      -1

ATGCTGTGGCAGGCACTTCGAAGATTTGGTCAAAAGCTGGTGCGGAGACGTTTGCTGGAGCTGGGCATGGGTGAAACCCGCCTTGCCAGA      90
M  L  W  Q  A  L  R  R  F  G  Q  K  L  V  R  R  R  L  L  E  L  G  M  G  E  T  R  L  A  R       30

TGCCTGAGCACTCTCGACTTAGTGGCCCTGGGTGTGGGCAGCACATTAGGTGCAGGTGTGTATGTCCTGGCTGGTGAGGTGGCCAAAGAG     180
C  L  S  T  L  D  L  V  A  L  G  V  G  S  T  L  G  A  G  V  Y  V  L  A  G  E  V  A  K  E       60
     +              +                   +                   +

AAAGCAGGACCCATCCATTGTGATCTGCTTTTTGGTGGCTGCTCTGTCCTCTGTGTTGGCTGGACTGTGTTATGCTGAGTTTGGTGCCCGG     270
K  A  G  P  S  I  V  I  C  F  L  V  A  A  L  S  S  V  L  A  G  L  C  Y  A  E  F  G  A  R      90

GTCCCCGGCTCTGGTTCTGCATACCTCTACAGTTATGTCACCGTAGGTGAACTCTGGGCCTTCACCACCGGCTGGAATCTCATTCTCTCC     360
V  P  G  S  G  S  A  Y  L  Y  S  Y  V  T  V  G  E  L  W  A  F  T  T  G  W  N  L  I  L  S      120

TACGTCATTGGTACAGCCAGTGTGGCCCGGGCTTGGAGCTCTGCTTTTGACAACCTGATTGGAAACCACATCTCTCAGACTCTGAAGGGG     450
Y  V  I  G  T  A  S  V  A  R  A  W  S  S  A  F  D  N  L  I  G  N  H  I  S  Q  T  L  K  G      150

ACCATCTTACTGAACATGCCTCATGTTCTTGCAGAATATCCAGATTTCTTTGCCTTGGCCCTTGTGTTGCTGCTCACTGGATTGCTGGTT     540
T  I  L  L  N  M  P  H  V  L  A  E  Y  P  D  F  F  A  L  A  L  V  L  L  L  T  G  L  L  V      180

TTGGGGGCTAATGAATCCGGCCTGGTTACCAAAGTGTTCACAGGGATGAACCTTTTGGTTCTCGGGTTTGTCATCATCTCTGGCTTCATT     630
L  G  A  N  E  S  G  L  V  T  K  V  F  T  G  M  N  L  L  V  L  G  F  V  I  I  S  G  F  I      210

AAGGGAGAGCTGAGAAACTGGAAGCTCACGAAAGAGGACTATTGCTTGACTATGAGTGAATCCAATGGCACTTGTAGCTTGGATTCTATG     720
K  G  E  L  R  N  W  K  L  T  K  E  D  Y  C  L  T  M  S  E  S  N  G  T  C  S  L  D  S  M      240
                                                             *

GGCTCTGGAGGTTTCATGCCTTTTGGCTTGGAAGGGATTCTCCGTGGCGCTGCTACCTGTTTCTATGCCTTCGTTGGTTTTGACTGTATT     810
G  S  G  G  F  M  P  F  G  L  E  G  I  L  R  G  A  A  T  C  F  Y  A  F  V  G  F  D  C  I      270

GCGACCACTGGGGAAGAGGCTCAGAATCCCCAGCGCTCCATCCCTATGGGTATTGTGATCTCACTGTCCATCTGCTTTTTGGCTTATTTT     900
A  T  T  G  E  E  A  Q  N  P  Q  R  S  I  P  M  G  I  V  I  S  L  S  I  C  F  L  A  Y  F      300

GGTGTGTCATCAGCACTGACGCTCATGATGCCTTACTACAAGCTTCAGCCTGAGAGCCCTCTGCCTGAAGCTTTTACCTATGTTGGCTGG     990
G  V  S  S  A  L  T  L  M  M  P  Y  Y  K  L  Q  P  E  S  P  L  P  E  A  F  T  Y  V  G  W      330

GAGCCTGCCCGCTACCTGGTGGCTATTGGCTCCCTCTGTGCACTTTCTACTAGCCTTTTGGGCTCTATGTTTCCTATGCCTCGGGTGATC     1080
E  P  A  R  Y  L  V  A  I  G  S  L  C  A  L  S  T  S  L  L  G  S  M  F  P  M  P  R  V  I      360

TATGCGATGGCAGAAGATGGCCTTCTGTTCCGTGTCCTTGCTAGGGTCCACAATGGCACACATACACCCATTGTGGCCACTGTGGTATCT     1170
Y  A  M  A  E  D  G  L  L  F  R  V  L  A  R  V  H  N  G  T  H  T  P  I  V  A  T  V  V  S      390

GGTGTTATTGCAGCATTCATGGCATTCCTCTTTGAACTCACTGATCTCGTGGACCTCATGTCAATCGGGACTCTGCTCGCTTACTCCCTG     1260
G  V  I  A  A  F  M  A  F  L  F  E  L  T  D  L  V  D  L  M  S  I  G  T  L  L  A  Y  S  L      420

GTGTCAATTTGTGTTCTTATCCTCAGATATCAGCCTGACCAAGAAATGAAGAACGGTGAAGAGGAAGTGGAATTGCAAGAGGAGAGGACA     1350
V  S  I  C  V  L  I  L  R  Y  Q  P  D  Q  E  M  K  N  G  E  E  E  V  E  L  Q  E  E  R  T      450

CTCGAAGCAGAAAAGCTGACTGTCCAGGCTCTATTTTGCCAAGTCGACTCCATCCCCACACTCCTTTCTGGCCGAATTGTCTATGTTTGC     1440
L  E  A  E  K  L  T  V  Q  A  L  F  C  Q  V  D  S  I  P  T  L  L  S  G  R  I  V  Y  V  C      480
                                                                                       =

TCATCACTGCTTGCTGTACTACTAACTGTTCTTTGCCTGGTGCTGACCTGGTGGACAACTCCGCTTCATTCTGGAGACCCAGTGTGGGTC     1530
S  S  L  L  A  V  L  L  T  V  L  C  L  V  L  T  W  W  T  T  P  L  H  S  G  D  P  V  W  V      510

ACAGTGGTTGTGCTGATCCTGGGGCTGATCCTTGGAATTTCTGGGGTCATCTGGAGACAACCACAGAACCGCACTCCCCTTCATTTTAAG     1620
T  V  V  V  L  I  L  G  L  I  L  G  I  S  G  V  I  W  R  Q  P  Q  N  R  T  P  L  H  F  K      540

GTGCCCGTTGTTCCTCTACTCCCACTGGTGAGCATCTTTGTGAATGTTTACCTGATGATGCAAATGACAGCTGACACTTGGGCCCGATTT     1710
V  P  V  V  P  L  L  P  L  V  S  I  F  V  N  V  Y  L  M  Q  M  T  A  D  T  W  A  R  F      570

GGGGTCTGGATGCTGATTGGATTTGCTATCTACTTCGGCTATGGGATCCAACACAGCGTGGAAGAAGTTAAGAATCATCAAACTCTACCC     1800
G  V  W  M  L  I  G  F  A  I  Y  F  G  Y  G  I  Q  H  S  V  E  E  V  K  N  H  Q  T  L  P      600

AAGACAAGGCCCCAAACTATAGACCTTGATCTCACCACTTCTTGTGTTCATAGTATTTGATGTAACTATACCCAAGTGTTGTCTTGTTCT     1890
K  T  R  P  Q  T  I  D  L  D  L  T  T  S  C  V  H  S  I  ***                               619

CCTACACAATAATGGAGAGTACTCTTGACCCCACGAAGAGCTGGGCCTCTCATGTGATGTTAGTGATGGATATGAAGTAGAGCTCTGTAT     1980

TTCTTGCGAAATGATGGCTAATTCTTTGCTGTTACTTTGTTTTTAACTTGTCCACTTTGAAATAGTCTCTGTAGTTGTTTCCTGGCTTTG     2070

AAAAATGTTATTAAAAGAAAATTGGGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                            2141
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B

periplasm

A potential N-glycosylation site

NH₂

cytoplasm

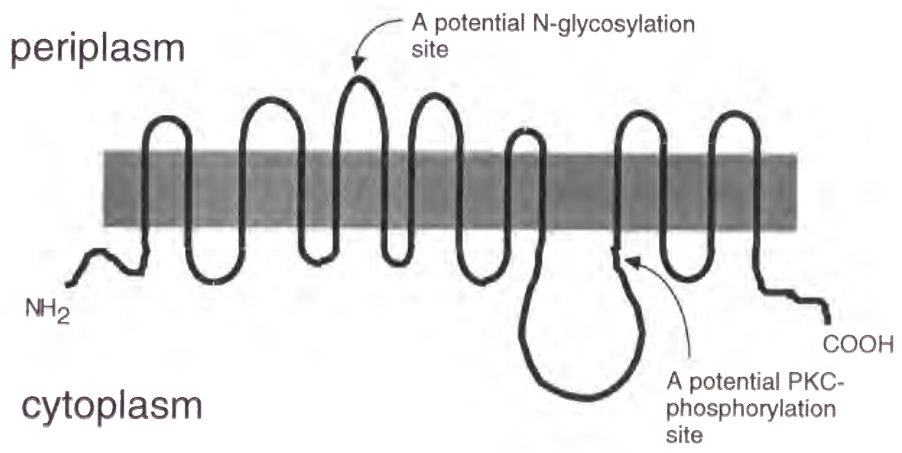A potential PKC-phosphorylation site

COOH

Fig. I - 1 Primary structure of rCAT3. **A.** Nucleotide and deduced amino acid sequences of rCAT3 cDNA. Nucleotides are numbered in the 5' to 3' direction beginning with the first methionine codon. *triple asterisk* - the stop codon franking the open reading frame, *underline* - putative transmembrane domains, *asterisk* - a potential N-linked glycosylation site, *double underline* - a putative PKC phosphorylation site, *plus* - a leucine zipper motif.
B. Schematic representation of the putative membrane topology of rCAT3 predicted by a hydrophobicity analysis. **C.** Homology alignments of rCAT3 and other CAT family proteins. Residues conserved in all members are boxed.

The predicted amino acid sequence of rCAT3 had a 53 - 58 % identity with those of other system y$^+$ transporters previously reported (Fig. I - 1C, GeneWorks™, IntelliGenetics). The leucine zipper motif is completely conserved in all members of the CAT family. Although individual sequences are variable, all of them have a potential N-glycosylation site and a potential PKC-phosphorylation site on the same extra- and intracellular loops, respectively. mCAT2a and b are believed to be alternative splicing products of the same gene and their sequences differ from each other only in a 41 amino acids stretch encoding the loop between the 8th and 9th TMs and a part of the 9th TM (a.a. 358 - 398 of mCAT2b). This divergent sequence has been shown to be responsible for the differential kinetics of L-arginine transport activity encoded by mCAT2a and b (I - 16). Within this stretch, the sequence of rCAT3 is homologous with those of the others in an order of rCAT1 > mCAT1 > mCAT2b > mCAT2a (GeneWorks™, IntelliGenetics).

System y$^+$ transport activity of rCAT3 - L-arginine transport activity of rCAT3 was tested by transient expression of the cDNA and L-[^{14}C]arginine incorporation assays in COS7 cells. In every experiment, one half of the plates were transfected with an empty vector plasmid and assayed in parallel. Basal incorporations exhibited by vector-transfected cells were 40 - 50 % of those exhibited by pMErCAT3-transfected cells. rCAT3-mediated incorporations were calculated as the differences between the values from pMErCAT3- and vector-transfected cells.

rCAT3-mediated incorporation of L-[^{14}C]arginine (100 nM) was time-dependent and was close to linear in the first 10 min (Fig. I - 2A). Therefore, the reaction time was set to 10 min in the following experiments. The rCAT3-mediated incorporation of L-[^{14}C]arginine was dose-dependent and was saturated over the concentration of 0.5 mM (Fig. I - 2B) as expected for a carrier-mediated process. Eadie-Hofstee plot analysis (Fig. I - 2B, inset) of the saturation isotherms gave half-saturation constant (Km) values of 103 ± 12 mM (means ± SEM, n=3).
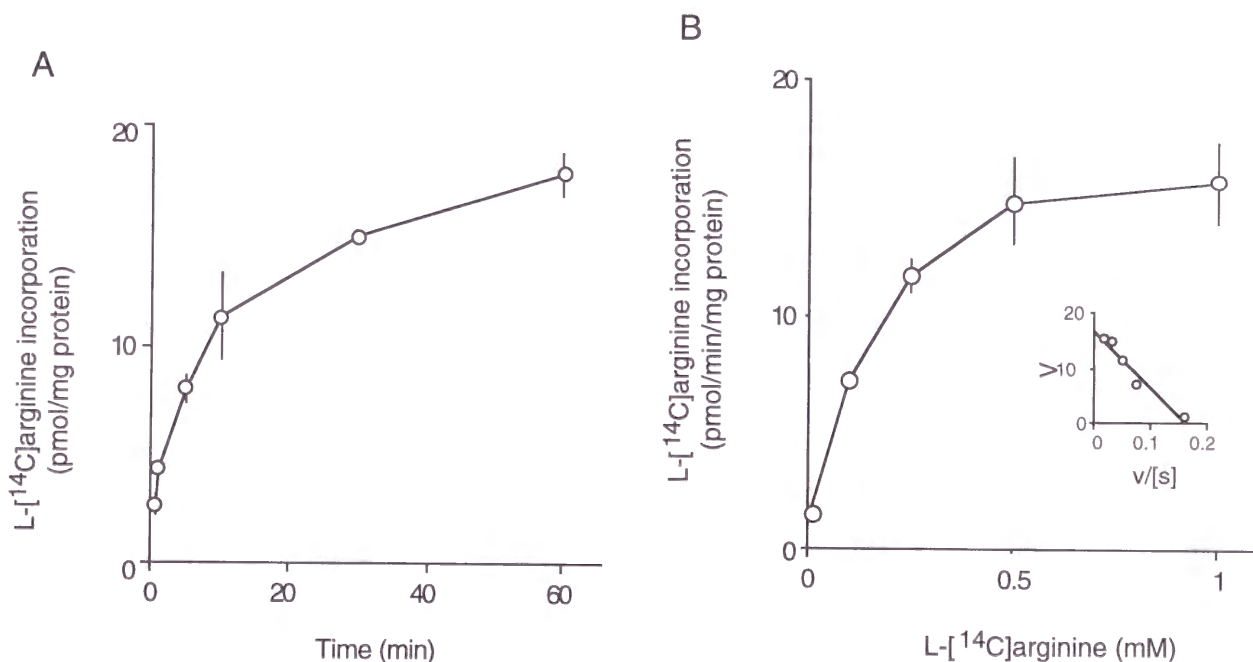
Fig. I - 2 Kinetics of rCAT3-mediated L-[^{14}C]arginine incorporation in COS7 cells. **A.** Time-course. COS7 cells transfected with pMErCAT3 or vector plasmid were incubated with L-[^{14}C]arginine (100 nM) for the time indicated and the radioactivity incorporated was determined as described in "Materials and Methods". The incorporation was normalized for the protein recovered from each well and the rCAT3-mediated incorporation was defined as the difference between the values from pMErCAT3- and vector-transfected cells. **B.** Saturation isotherms. COS7 cells transfected with pMErCAT3 or vector plasmid were incubated for 10 min with increasing concentrations of L-[^{14}C]arginine. To obtain concentrations higher than 100 nM, L-[^{14}C]arginine (100 nM) was diluted with unlabeled L-arginine and the radioactivities recovered were normalized for the reduced specific activities. rCAT3-mediated incorporation was determined as described above. *Inset* is an Eadie-Hofstee plot of the same data. Both in **A.** and **B.**, shown are the means ± SEM of triplicate determinations obtained in a single experiment. These experiments were replicated three times with similar results.

The Km values obtained were comparable with those of the L-arginine incorporation in *Xenopus* oocytes mediated by mCAT1 (I - 14) or mCAT2b (I -

16), both of which were verified to encode a high affinity system y+ transporter. They were also comparable with those for the system y+ transport activity determined in brain slices, cultured neurons and glial cells (I - 10,11). In addition, they were at least twice as low as those of the L-arginine incorporation in *Xenopus* oocytes mediated by mCAT2a (I - 18), which encodes a liver-specific, low affinity transporter distinct from system y+.

The substrate specificity was examined by testing the ability of unlabeled amino acids to inhibit the rCAT3-mediated L-[14C]arginine incorporation. Of the twenty-two naturally-ocurring L-amino acids, only the three basic amino acids, L-arginine, L-lysine and L-ornithine, competed the incorporation (Fig. I - 3). Both of the two structural analogues of L-arginine tested, D-arginine and L-citrulline, also caused a significant inhibition of the incorporation (Fig. I - 3).
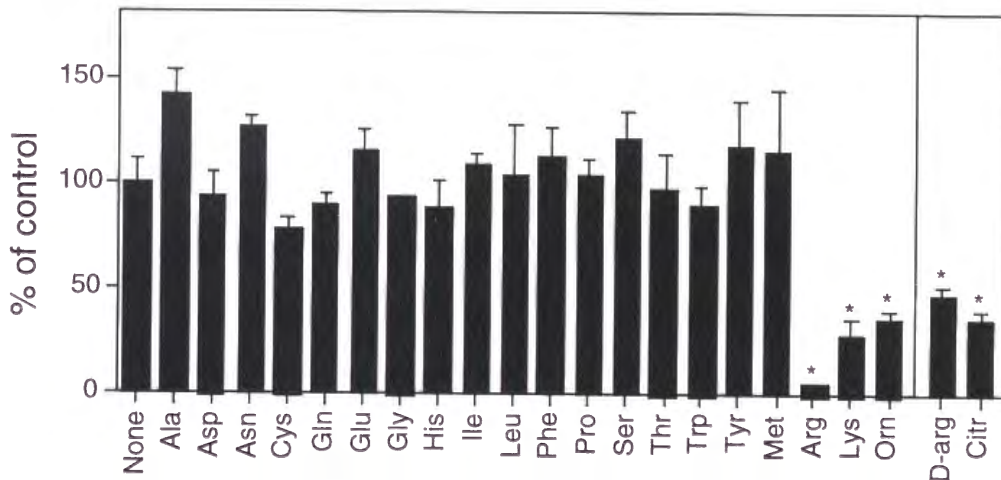
Fig. I - 3 Inhibition of rCAT3-mediated L-[14C]arginine incorporation in COS7 cells by cationic amino acids. COS7 cells transfected with pMErCAT3 or vector plasmid were incubated for 10 min with L-[14C]arginine (100 nM) in the absence (None) or presence of the individual amino acid indicated (1 mM). All of the amino acids applied were L isomers except for D-arginine. rCAT3-mediated incorporation was determined as described in the legend to Fig. I - 2. The data are presented as relative to the incorporation in the absence of the unlabeled amino acid. Each bar represents the means ± SEM of 3 determinations each done in triplicate. * p < 0.01, significantly different from the control values (ANOVA).

There was, however, a statistically significant difference between the effects of L and D isomers of arginine, suggesting a stereospesficity of the incorporation. To demonstrate that the inhibitory effects of the cationic amino acids observed

were due to direct competition for rCAT3, we conducted incorporation experiments of redioactive L-lysine and L-ornithine (Fig. I - 4).
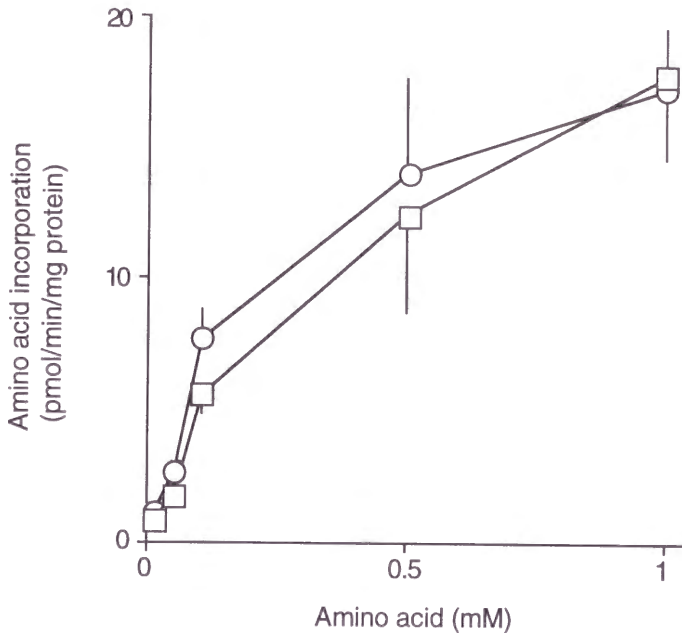

Fig. I - 4 Dose-dependence of rCAT3-mediated incorporations of L-[^{3}H]lysine (circles) or L-[^{14}C]ornithine (squares) in COS7 cells. The assays were performed exactly as described for L-[^{14}C]arginine in the legend to Fig. I - 2B. The reaction time was 10 min. Shown are the means ± SEM of triplicate determinations obtained in a single experiment. These experiments were replicated three times with similar results. The Km values obtained were given in the text.

In both cases, the rCAT3-mediated incorporations were dose-dependent and Eadie-Hofstee plot analysis (not shown) gave Km values of 147 ± 22 and 219 ± 26 mM (means ± SEM, n=3) for incorporations of L-[^{3}H]lysine and L-[^{14}C]ornithine, respectively. The specificity for cationic amino acids, together with the high affinity for L-arginine, indicated a system y$^+$ transport activity of rCAT3.

Next we examined the dependence of the rCAT3 activity on extracellular monovalent ions and membrane potentials by manipulating the ionic constitutions of the extracellular medium. Neither isotonic substitution of Na$^+$ with Li$^+$ nor that of Cl$^-$ with CH$_3$COO$^-$ caused any change in the rCAT3-mediated L-[^{14}C]arginine incorporation (data not shown). In contrast, substitution of Na$^+$ with K$^+$ caused a dose-dependent inhibition of the rCAT3 activity (Fig. I - 5).
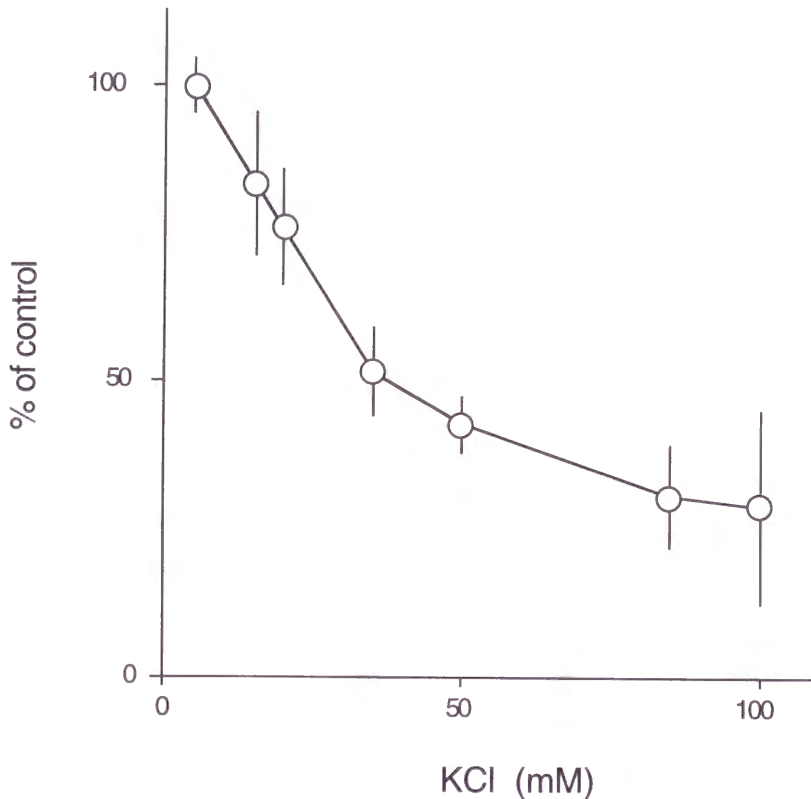
15

Fig. I - 5 Inhibition of rCAT3-mediated L-[^{14}C]arginine incorporation in COS7 cells by high concentrations of K$^+$. COS7 cells transfected with pMErCAT3 or vector plasmid were incubated for 10 min with L-[^{14}C]arginine (100 nM) in HBS containing increasing concentrations of K$^+$. Normal HBS contained 5 mM KCl and 150 mM NaCl. To increase the concentrations of K$^+$, NaCl was replaced with equimolar KCl. rCAT3-mediated incorporation was determined as described in the legend to Fig. I - 2. The data are presented as relative to the incorporation in normal HBS. Shown are the means ± SEM of 3 determinations each done in triplicate.

These results are consistent with the functional properties of system y$^+$ described previously, i.e., it is dependent neither on the extracellular Na$^+$ nor Cl$^-$ (I - 9) and is inhibited by high K$^+$-induced membrane depolarization (I - 9,30,31).

Inhibition of rCAT3-mediated L-[^{14}C]arginine transport by NOS inhibitors - Currently available NOS inhibitors are structural analogues of L-arginine and some of them also inhibit L-arginine transport (I - 10,32). Therefore, we examined the ability of three drugs, L-NMMA, L-NIO and L-NAME to inhibit rCAT3-mediated L-[^{14}C]arginine incorporation in COS7 cells (Fig. I - 6).
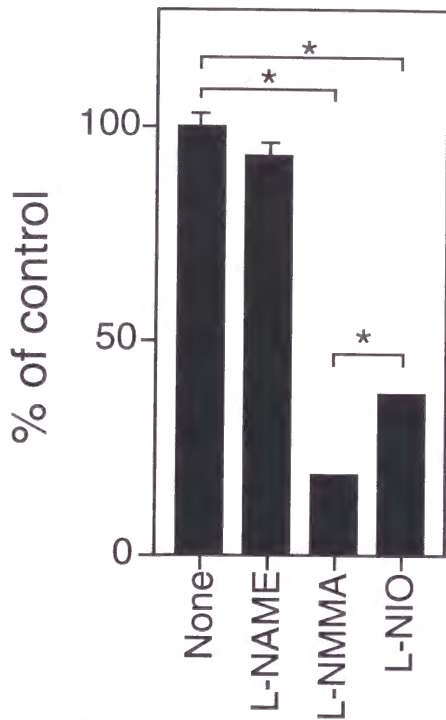
Fig. I - 6 Inhibition of rCAT3-mediated L-[^{14}C]arginine incorporation in COS7 cells by NOS inhibitors. COS7 cells transfected with pMErCAT3 or vector plasmid were incubated for 10 min with L-[^{14}C]arginine (100 nM) in the absence or presence of the individual NOS inhibitor (1 mM). rCAT3-mediated incorporation was determined as described in the legend to Fig. I - 2. The data are presented as relative to the incorporation in the absence of the drug. Each bar represents the means ± SEM of 3 determinations each done in triplicate. * p < 0.01, significantly different from each other (ANOVA).

When the transfected cells were exposed to L-[^{14}C]arginine (100 nM) in the presence or absence of the NOS inhibitors (1 mM), both L-NMMA and L-NIO caused a significant inhibition of the rCAT3-mediated L-[^{14}C]arginine incorporation, while L-NAME caused only a marginal effect. There was also a statistically significant difference between the effects of L-NMMA and L-NIO. Thus, the rank order of potency of the three drugs was L-NMMA > L-NIO >> L-NAME. Similar rank order of potency was observed for these drugs to inhibit system y+ L-arginine transport in endothelial cells (I - 32) and in neuronal cells (I - 10). These results gave further evidence for the system y+ transport activity of rCAT3 and support the notion that the transport of L-NAME is mediated by a system different from y+ (I - 32).

Brain-specific expression of rCAT3 mRNA - On northern blot of poly (A)+ RNA from rat various tissues, a single band with a size of 3.3 kb was detected with a

rCAT3 cDNA probe only in brain (Fig. I - 7), suggesting the brain-specific expression of rCAT3.
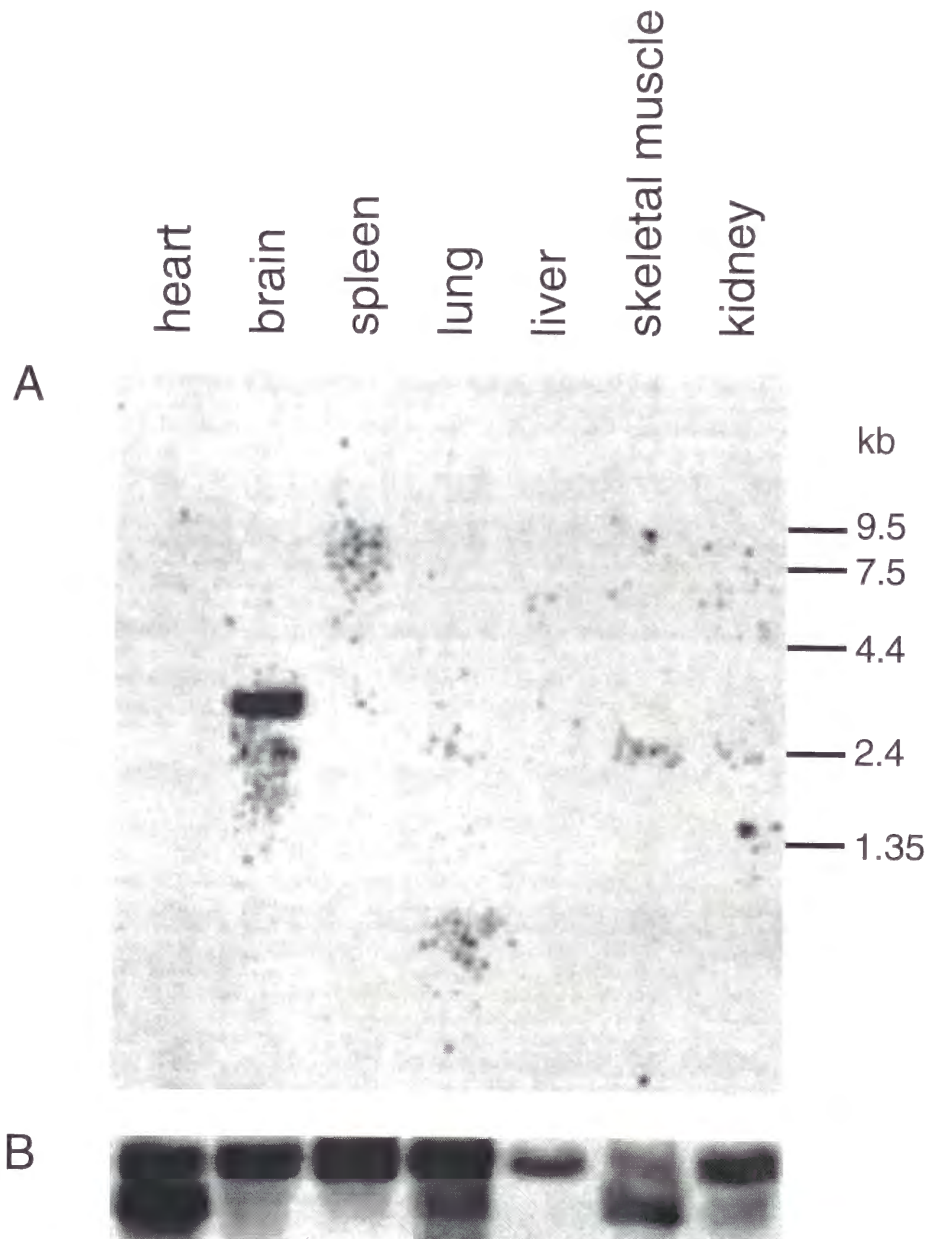
A

Fig. I - 7 Brain-specific expression of rCAT3 mRNA. Northern blotting was performed as described in "Materials and Methods". **A.** An autoradiograph showing the hybridization signal of a rCAT3 cDNA probe on a rat multiple tissue northern blot membrane (Clontech). Molecular sizes are given on the right. **B.** Internal control. The same blot was probed with a human b-actin cDNA.

Although direct evidence is lacking, it is very likely that the brain-specific 3.4 Kb hybridization signal of rCAT1 detected by Wu et al. (I - 20) was rCAT3.

Conclusion - We have isolated a cDNA clone rCAT3 which encodes a novel member of the murine CAT family. Expression and functional characterization of the gene verified a high-affinity, system y+ transporter activity of rCAT3. The expression of rCAT3 mRNA is restricted to brain. The rCAT3 cDNA will be an essential tool to further clarify the molecular basis of the system y+ activity in the nervous tissues and that of the regulation of NO synthesis by L-arginine transport.

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Part II   **Physiological Role(s) of rCAT3**

SUMMARY

Recently I cloned a brain specific cationic amino acid transporter (rCAT3) from rat brain. In the present study, spatial patterns of expression of rCAT3 mRNA and the corresponding mRNA was examined in the adult rat brain by in situ hybridization analysis. The mRNA of rCAT3 was expressed in all over brain, and strongly condensed at hypothalamus, High magnificent analysis showed rCAT3 mRNA was restricted in neuronal cells. These facts indicated that L-arginine mediated hypothalamic neuronal circuit was dependent of rCAT3. Furthermore, the effects of neurite extension on B103 neuronal cell line by L-arginine transport was examined. Northern blot analysis showed rat neuronal B103 line highly expressed rCAT3 mRNA. L-arginine incorporation activity of B103 was shown in dose-response manner and its pharmacological profile was same as rCAT3. Neurite sprouting of B103 by dbcAMP depends upon extracellular L-arginine, but 8-Br-cGMP treatment causes neurite sprouting on B103 in L-arginine depletion medium. To clarify whether neurite sprouting activity depends on rCAT3 mediated L-arginine incorporation or not, I examined pharmacological profile of neurite sprouting on B103 cells. Neurite sprouting activity on B103 cells were inhibited by cationic amino acid L-lysine or L-ornithine, or L-arginine analogs, L-NMMA or L-NIO, but not L-NAME. These profile is a good agreement with rCAT3 mediated L-arginine incorporation. For disturb rCAT3 mediated L-arginine incorporation, antisense oligonucleotide of rCAT3 treatment showed significantly reduced the rate of neurite sprouting on B103 cells. These results indicated that neurite outgrowth of B103 by dbcAMP depended on L-arginine incorporation mediated by rCAT3.

INTRODUCTION

Nitric Oxide(NO)[1], a highly diffusible molecule involved in signal transduction in brain, is formed from the terminal guanidino group of L-arginine by an enzyme NO synthase (NOS) . Of the two classes (constitutive and inducible) of NOS isoforms, neuronal NOS belongs to the constitutive type and it is generally accepted that calcium/calmodulin signaling is the primary mechanism to regulate its activity(II - 1). Several lines of evidence suggested that, besides the regulation of NOS activity, NO synthesis is also regulated by availability of the substrate L-arginine (II - 2) and that L-arginine is provided to NOS via transport from the extracellular space rather than from the intracellular metabolic pool. For example, N-methyl-D-aspartate-induced NO production in neuroblastoma cells depended on extracellular L-arginine(II - 3). Inhibitory effects of these cationic amino acids on NO synthesis were also observed in brain synaptosomes(II - 4). Furthermore, exogenous L-arginine enhanced NO synthesis in brain slices(II - 5).

The amino acids are incorporated from exterior medium to intracellular space via carrier proteins on the cell surface. Among the several types of amino acid transport systems distinguished by substrate specificity, cationic amino acids such as L-arginine, L-lysine and L-ornithine share the same transporter with a sodium-independent activity, called system y^+(II - 6). Although the presence of a system y^+ activity in neuronal / glial cells has been demonstrated by functional studies (II - 7,8).

Recently I cloned brain specific cationic amino acid transporter named as rCAT3. rCAT3 shows that high affinity system y^+ activity, and its mRNA expression restricted in brain(II - 9). By RNase protection assay, Stoll et al.(II - 10) demonstrated a marked enrichment of the rat CAT1 (rCAT1) mRNA in brain capillary, suggesting a role of rCAT1 in amino acids transport by endothelial cells at the blood brain barrier. Therefore I tried to examine sub

localization of rCAT3 in brain and examined NO synthesis was regulated by rCAT3 mediated L-arginine transport in brain. The amino acids are incorporated from exterior medium to intracellular space via carrier proteins on the cell surface. Among the several types of amino acid transport systems distinguished by substrate specificity, cationic amino acids such as L-arginine, L-lysine and L-ornithine share the same transporter with a sodium-independent activity, called system y^+(II - 6). Although the presence of a system y^+ activity in neuronal / glial cells has been demonstrated by functional studies (II - 7,8).

Recently I cloned brain specific cationic amino acid transporter named as rCAT3. rCAT3 shows that high affinity system y^+ activity, and its mRNA expression restricted in brain(II - 9). By RNase protection assay, Stoll et al.(II - 10) demonstrated a marked enrichment of the rat CAT1 (rCAT1) mRNA in brain capillary, suggesting a role of rCAT1 in amino acids transport by endothelial cells at the blood brain barrier. This report lead us to examine the idea that the expression of rCAT3 was restricted in neuronal cells. Therefore I tried to examine sub localization of rCAT3 in brain and examined NO synthesis was regulated by rCAT3 mediated L-arginine transport in brain.

Nitric oxide (NO), a diffusible and unstable gas, has been implicated in inter- and intra-cellular communication in the nervous system(II - 11). NO also plays a role in neural development, plasticity and alterations of synaptic function such as long-term potentiation and long-term depression some of which likely involve growth and remodeling of neurites. Some actions of NO are mediated directly by protein modification (e.g., nitrosylation) and others by activation of soluble guanylate cyclase (soluble GC), which increases intracellular levels of guanosine 3',5'-cyclic monophosphate (cGMP). NO is synthesized by the enzyme nitric oxide synthase (NOS) from L-arginine, which is induced by treatment of CNS neurons or pheochromocytoma PC12 cells

with NGF(II - 12). Treatment of NO donor on primary culture of neuron or NGF treated PC12 cells increased the proportion of neurite positive cell number via cGMP - G-kinase pathway(II - 13).

Several lines of evidence suggested that, besides the regulation of NOS activity, NO synthesis is also regulated by availability of the substrate L-arginine and that L-arginine(II - 14) is provided to NOS via transport from the extracellular space rather than from the intracellular metabolic pool. For example, N-methyl-D-aspartate-induced NO production in neuroblastoma cells depended on extracellular L-arginine(II - 15) . Inhibitory effects of these cationic amino acids on NO synthesis were also observed in brain synaptosomes(II - 16). Furthermore, exogenous L-arginine enhanced NO synthesis in brain slices(II - 17).

These reports leads us rCAT3 may regulate neurite sprouting as controlling NO synthesis in neuronal cells. Here I reported evidence that neurite outgrowth depends on extracellular L-arginine incorporation and extracellular L-arginine was transported by neuron specific cationic amino acid transporter rCAT3.

MATERIALS & METHODS

RNA probes - *EcoRI* - *BamHI* fragment or *EcoRI* fragment of rCAT3 cDNA was subcloned into pBluescript II (KS-) (pT1 or 2). [^{35}S] labeled riboprobes were prepared by *in vitro* transcription of the EcoRI digested pT1 or HindIII digested pT2 by using T7 or T3 RNA polymerase (Stratagene, La Jolla CA) with a-[^{35}S] uridine triphosphate (Dupont NEN, Natick MA). The incorporation of [^{35}S] - UTPs were determined by liquid scintillation counter, and specific activities of each probes were usually ~ 1 x 10^9 cpm / µg synthesized riboprobes. For preparation of cold riboprobes, in vitro transcription of same templates with S - uridine triphosphate (Dupont NEN, Natick CA). All probes were hydrolyzed ~ 200 bp before use. To examine the specificity of riboprobes, Northern blot analysis was carried out. Only single band with a size of 3.4 kb, corresponded to rCAT3, was detected in the total RNA from adult rat brain.

In situ hybridization Histochemistry - Animals are decapitated and the brains were quickly removed and frozen with liquid nitrogen. The brains embed in OCT compounds (Miles IN), cut in a cryostat at a thickness of 10µm, thaw-mounted onto APS coated slides (Matsunami, Tokyo Japan), and stored at -80 C until used. The sections were fixed with 4% formaldehyde in Dulbecco's phosphate buffered saline (PBS) for 10 min at room temperature, washed with PBS for 10 min twice, acetylated with 0.25 % acetic anhydrite, 0.1 M triethanolamine in 0.9 % NaCl for 10 min, dehydrated with 70 % ethanol for 3 min, 95 % ethanol for 3 min, 100 % ethanol for 3 min, and 100 % ethanol for 3 min, dried, and stored in - 80 °X. Τηεν tissue sections were hybridized at 60 C for 6h in 50 % formamide, 2 x SSC (SSC is 17 mM sodium citrate, pH 7.0, plus 150 mM NaCl), 10 mM Tris pH 7.4, 1x Denhardt's (containing 0.02 % BSA fraction V, 0.02 % polyvinylpyrolydone, 0.02 % Ficoll 400), 10 % Dextran Sulfate, 0.2 % SDS, 250 µg / ml Baker's yeast tRNA (Boheringer-Manheim),

500 μg / ml salmon sperm DNA (Sigma), 100 mM DTT, and 1 x 10⁵ cpm / μl of [^{35}S] labeled with or without S labeled riboprobes. After hybridization, the sections were washed with 2 x SSC with 10 mM b-mercaptoethanol for overnight, 2 x SSC with 10 mM b-mercaptoethanol for 1h at 60 C, treated with RNaseA (20 μg / ml RNaseA, 500 mM NaCl, 10 mM Tris, 1 mM EDTA (pH 7.5)) for 10 min at 37 C, rinsed with 1 x SSC, washed with 0.1 x SSC containing 10 mM b-mercaptoethanol for 1h at 60 C, and dehydrated in 75 % ethanol containing 0.3 M ammonium acetate for 3min, 95 % ethanol containing 0.3 M ammonium acetate for 3min, 100 % ethanol for 3min, air dried. The hybridized probes were visualized by exposing to b-MAX film (Amersham) or emulsion autoradiograhy by dipping with NTB-2 (Kodak). Each sections were counter stained by nissel.

Northern blotting - The probe was prepared by labeling the *EcoRI* fragment of pBSKrCAT3 with [^{32}P]dCTP (110 TBq/mmol, Amersham) exactly as described above. Total RNA isolation of B103, C6, PC12, and 293 rat cell lines were prepared by acid guanidine phenol chloroform method. Blotting procedures were as described (II - 23). The blot was visualized for radioactivity with BAS2000 image analyzer (Fuji, Tokyo, Japan).

L-arginine transport assay - The cells were washed twice with HEPES-buffered saline (HBS) (150 mM NaCl, 10 mM HEPES pH 7.5, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM KCl) and further incubated in HBS at 37 °C for 10 min. The reaction was started by changing the media to HBS containing L-[^{3}H]arginine (35 - 70 Ci/mmol). The concentrations of L-[^{14}C]arginine applied and the reaction time were indicated in the figure legends. The reaction was stopped by washing the cells three times with ice-cold HBS. The cells were then lysed in 0.2 N NaOH/1% sodium dodecyl sulfate and the radioactivity incorporated

was determined by a solid scintillation counter. The radioactive amino acids were obtained from Amersham. Protein concentration of the cell lysate was determined using a MicroBCA kit (Pearce, Rockford, IL). All of the unlabelled amino acids and the three NOS inhibitors (N^G-monomethyl-L-arginine [L-NMMA], L-N^5-(1-iminoethyl)-ornithine [L-NIO], N^G-nitro-L-arginine methyl ester [L-NAME]) used for the inhibition experiments were obtained from Sigma (St. Louis, MO).

The Neurite Extension Assay - B103 cell clone (kindly provided from Dr. schubert D (II - 18)) were maintained with Dulbecco's modified eagle medium (DMEM) supplement with 10% fetal bovine serum in humidified atmosphere containing 5% CO_2 incubator. For the experiments in serum free condition, late exponentially dividing B103 cells were washed fifth with PBS, dissociated by gentle trituration in PBS, spun down, brought up in low L-arginine concentration MEM using MEM select-amine kit with manufacture's instruction (GIBCO BRL). B103 cells were seeded 5 x 10^3 cells of 100μl /well in multiwell plate and cultured for 40 hr. under various condition displayed in figures. Cells were maintained in humidified chamber maintained at 37°C, 5% CO_2. At the end of the experiment, the culture were fixed by carefully adding 200μl of Dulbecco's PBS (CMF) containing 0.33M sucrose and 4% paraformaldehyde. After fixation for several hours, the fixative was carefully removed and staining with 0.5% CBB R-250. After staining for several minutes, culture were washed three times with DW. Cultures were observed on Zeiss Axiobert with manufacture's instruction. Each experimental condition was in triplicate. Routinely, I photographed the central portion of each well with best phase contrast, excluding small portion of the cell and cell aggregates. A neurite was defined as a cellular process longer than the twice of longest diameter of the cell body. The sum of neurite lengths was then

divided by the number of cells counted. At least 200 cells were counted in each experimental conditions.

Antisense and sense oligonucleotide treatment - A 20-mer antisense oligonucleotide complement of the 5`-region of BAT mRNA containing the initiator AUG codon and the corresponding sense oligonucleotide were purchased from genosys by the phosphorothioate approach using tetraethylthiuram disulfide(II - 19) . Sequences of antisense and sense oligonucleotides were 5' - CGAAGTGCCTGCCACAGCAT - 3' (complement of nt 1-20) and 5' - ATGCTGTGGCAGGCACTTCG - 3' (nt 1-20), respectively. The nucleotide numbers are according to the first letter of the initiator codon is numbered +1.

RESULTS & DISCUSSION

rCAT3 expressed in all over brain, especially condensed in thalamus -
Previously, I identified brain specific cationic amino acid transporter rCAT3
from rat brain. Expression studies showed that rCAT3 displayed system y+
cationic amino acid transporter activity. Northern blot analysis of rCAT3
showed that rCAT3 expression was restricted in brain. To identify more clearly
the cellular localization of RNAs hybridizing to rCAT3 cDNA fragment,
paraformaldehyde fixed rat sagital or colonal sections were hybridized with
[^{35}S] labeled rCAT3 cDNA fragment. Figure 1 reveal that rCAT3 mRNA is
expressed in adult brain. Prominent hybridization signals were observed over
sub populations of putative hypothalamus neurons. week signals are
obtained all over brain. moderate signals were obtained pyramidal cell layer
of hippocampus, mitral cell layer of olfactory bulb, and granule cell layer of
cerebellum cortex. Strong positive signals were obtained in reticular nucleus
and mammary nucleus in thalamus (Fig. II - 1 A, C, and D). In contrast, No
significantly signals were obtained in control sections (Fig. II - 1 B and E).
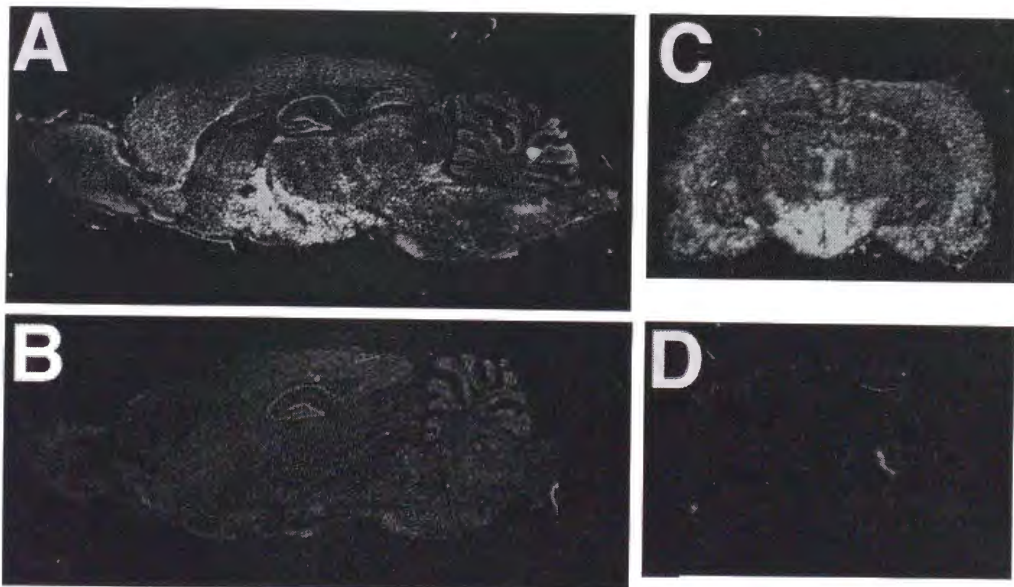
Fig. II - 1   Distribution of rCAT3 mRNA expression by in situ hybridization histochemistry
A sagital section (A and B) or colonal section (C and D) of adult rat brain were hybridized with [^{35}S] - UTP
labeled antisense probe to coding region of rCAT3 and exposed to β-MAX file for a week. Note prominent
labeling of the hypothalamus. A parallel sagital (B) or coronal (D) section was hybridized with [^{35}S] - UTP
labeled antisense probe and excess non labeled probe showed insignificant labeling. No significant

31

hybridization signals were obtained hybridizing with [^{35}S] - UTP labeled sense probe or RNase A pre-treatment (data not shown).

The present In situ hybridization histochemical analysis showed that rCAT3 mRNA is expressed in only neuronal cells but not satellite cells. Previous studies showed that a high affinity L-arginine transport activity in brain slice(II - 10) or cultured neuron(II - 7) were existed, but RNA protection analysis showed that CAT1 gene was expressed in BBB(II - 10). Our results revealed rCAT3 mediates L-arginine transport in neuronal cells in brain.

rCAT3 mRNA restricted in neuronal cells but not satellite cells - To identify the rCAT3 positive cells are neuronal or satellite cells, I analysis in high magnificent by exposing by emulsion auto radiography (Fig. II - 2).
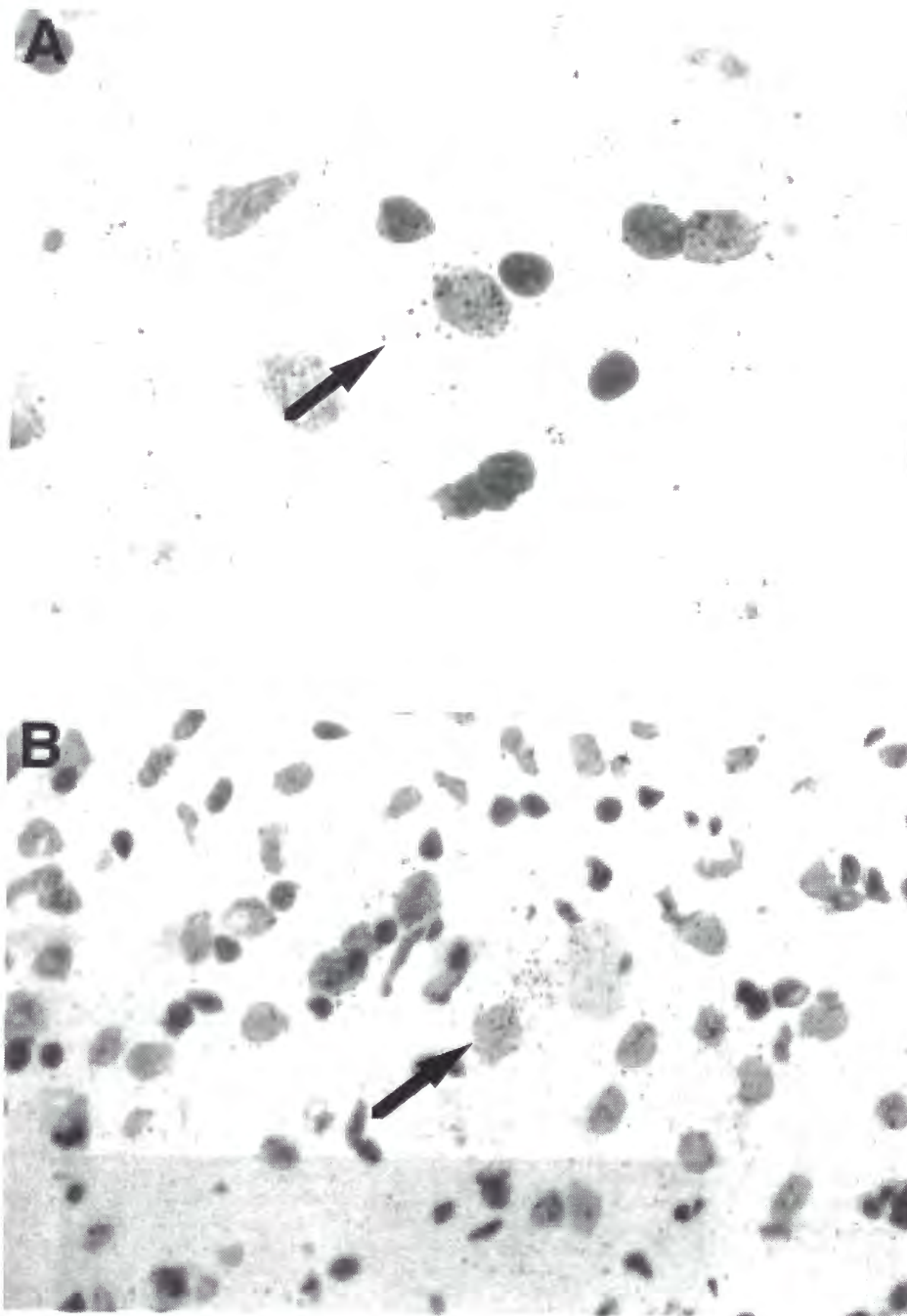
Fig. II - 2   Localization of rCAT3 mRNA expression by in situ hybridization histochemistry
A hybridized sections are exposed by emulsion autoradiogralhy and counter stained by nissel. In cortical layer, the signals of rCAT3 probes were detected near the neuronal cells (allow) but not satellite cells (A, B).

rCAT3 hybridization signals were detected at only neuronal cell but not glial cells or blood vessels (Fig. II - 2A and 2B).

Expression of rCAT3 mRNA was especially condensed in thalamus. In thalamic neuron, physiological sensory input caused L-arginine release(II -

20,21). This L-arginine release may dependent upon rCAT3 because L-arginine transport via rCAT3 was affected by membrane potential(II - 9). The L-arginine release in thalamus enhances L-glutamate - nitric oxide dependent neuronal activity in vetrobasal thalamic neuron. rCAT3 may controls the local nitric oxide levels in hypothalamus.

rCAT3 expression in rat neuronal B103 line - rCAT3 mRNA was expressed in brain tissue by northern blot analysis previously. To determine the specificity of rCAT3 mRNA expression in various brain derived cell line, northern blot analysis was occurred. The 3.4 kb hybridization signals corresponded to rCAT3 was obtained in C6, PC12, and B103 cell line, but not human kidney cell line(Fig. II - 3).
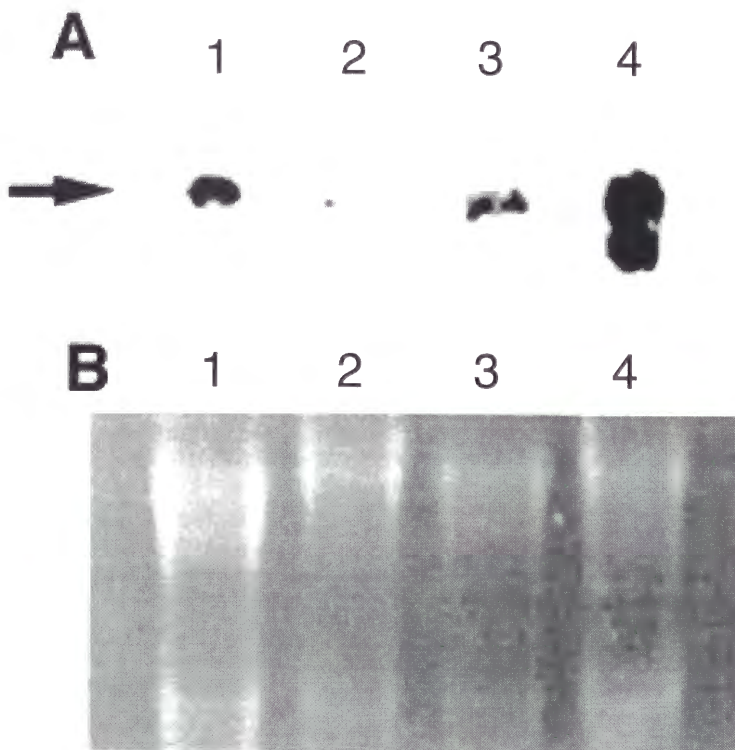
Fig. II - 3   Northern blot analysis of rCAT3 mRNA expression in various cell lines
(A) Total RNA (20µg per lane) isolated from C6 (lane 1), 293 (lane 2), PC12 (lane 3), and B103 (lane 4) rat cell line were separated on formaldehyde - agarose gel, blotted, and hybridized with [35S] - UTP labeled antisense probe. The auto radiogram was visualized with BAS2000. The hybridized transcripts is ~ 3.4 kb. (B) RNA levels were normalized with rRNA. Total RNA (20µg per lane) isolated from various rat cell

lines was separated on formaldehyde - agarose gel and stained by ethidium bromide. Same results were obtained by hybridization using human GAPDH probe.

In B103, rCAT3 mRNA hybridized signal was condensed. In 293, as a negative control, specific hybridized signal was not detected at all. B103, isolated from rat central nervous system, was known that B103 have several neuron like phenotype, especially, sprouts neurite responded by dbcAMP stimulation. rCAT3 mRNA was expressed in C6 glioma, PC12 phenocrocytoma, and B103 neuronal cell line but not 293 human kidney line. In B103 line, rCAT3 mRNA was expressed highly than C6 or PC12 cells. In brain slices, a high affinity system y+ cationic amino acid transport activity was existed, and CAT1 and rCAT3, a high affinity system y+ transporter, mRNA was detected by northern blotting. But, previous study of CAT1 mRNA expression showed that CAT1 mRNA expression was restricted in blood brain barrier (BBB)(II - 22). Therefore, rCAT3 may expresses in non BBB consisted cells, such as neuron.

To further examine of rCAT3 expression in B103, we determine L-arginine transport activity on B103 cells.
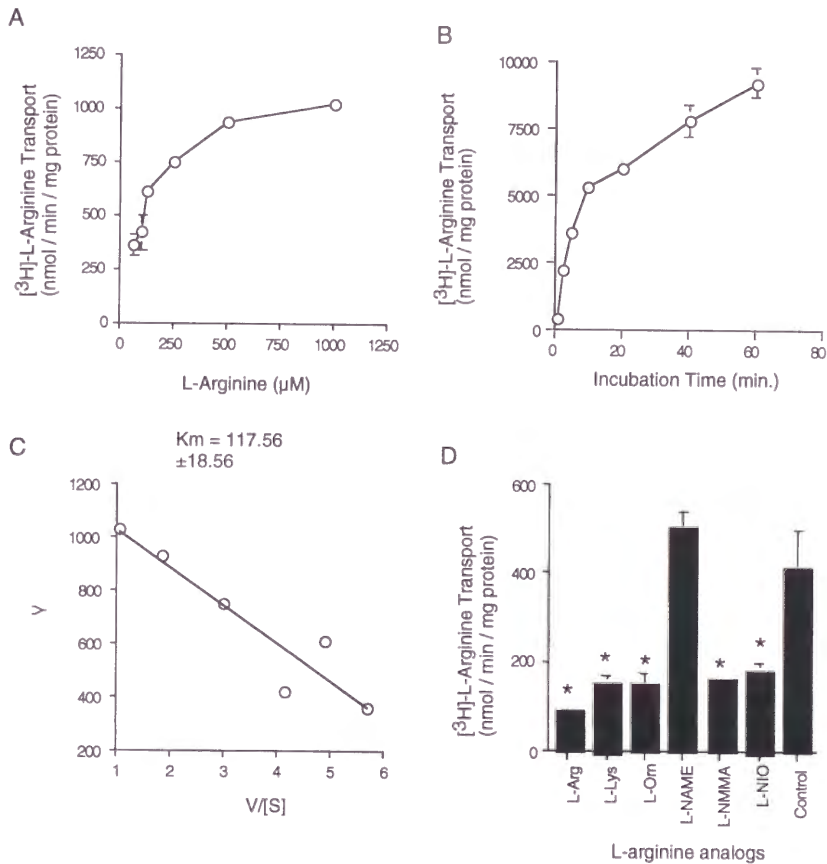
Fig. II - 4   L-Arginine transport activity by B103
(A) Saturation isotherms. B103 were incubated for 10 min. with increasing concentrations of L-[³H]arginine. To obtain concentrations higher than 100 nM, L-[³H]arginine (100 nM) was diluted with unlabelled L-arginine and the radioactivities recovered were normalized for the reduced specific activities. rCAT3-mediated incorporation was determined as described above. (B) Time-course. B103 were incubated with L-[³H]arginine (100 µM) for the time indicated and the radioactivity incorporated was determined as described in "Materials and Methods". The incorporation was normalized for the protein recovered from each well. (C) An Eadie-Hofstee plot of the same data. The Km value was indicated. (D) Inhibition of L-[³H]arginine incorporation in B103 cells by cationic amino acids. B103 cells were incubated for 10 min. with L-[³H]arginine (100 µM) in the absence (None) or presence of the individual amino acid indicated (1 mM). Both in (A), (B). and (D). shown are the means ± SEM of triplicate determinations obtained in a single experiment. These experiments were replicated three times with similar results. * ; p < 0.05, significantly different from the basal increase (non paired Student's t test).

L-arginine transport activity in shown in fig. II - 4. Incorporation of L-

[³H]arginine (100 µM) was time-dependent and was close to linear in the first

10 min. (Fig. II - 4B). Therefore, the reaction time was set to 10 min. in the following experiments. The rCAT3-mediated incorporation of L-[^{3}H]arginine was dose-dependent and was saturated over the concentration of 0.5 mM (Fig. II - 4A) as expected for a carrier-mediated process. Eadie-Hofstee plot analysis (Fig. II - 4C) of the saturation isotherms gave half-saturation constant (Km) values of 117 ± 18 μM (means $\pm$ SEM, n=3). The Km values obtained were comparable with those of the L-arginine incorporation in rCAT3 was verified to encode a high affinity system y$^+$ transporter. They were also comparable with those for the system y$^+$ transport activity determined in brain slices, cultured neurons and glial cells .

The substrate specificity was examined by testing the ability of unlabelled amino acids to inhibit the L-[^{3}H]arginine incorporation on B103. The three basic amino acids, L-arginine, L-lysine and L-ornithine, competed the incorporation (Fig. II - 4D). These system *y$^+$* specificity is very similar to the profile of rCAT3 mediated L-arginine incorporation. Currently available NOS inhibitors are structural analogs of L-arginine and some of them also inhibit L-arginine transport. Therefore, I examined the ability of three drugs, L-NMMA, L-NIO and L-NAME to inhibit L-[^{3}H]arginine incorporation in B103 cells (Fig. II - 4D). When B103 cells were exposed to L-[^{3}H]arginine (100 μM) in the presence or absence of the NOS inhibitors (1 mM), both L-NMMA and L-NIO caused a significant inhibition of the L-[^{3}H]arginine incorporation, while L-NAME caused only a marginal effect. There was also a statistically significant difference between the effects of L-NMMA and L-NIO. Thus, the rank order of potency of the three drugs was L-NMMA > L-NIO >> L-NAME. Similar rank order of potency was observed for these drugs to inhibit system y+ L-arginine transport in neuronal cells. These results gave further evidence for the system y$^+$ transport activity of rCAT3 and support the notion that the transport of L-NAME is mediated by a system different from y+.

Functional studies of L-arginine transport in B103 cells indicated that a high affinity system y+ cationic amino acid transport activity was existed. At least two genes, mCAT1 (mouse cationic amino acid transporter 1), mCAT2 have been identified and verified to encode a high affinity (Km = ~100 mM) system y$^+$ activity in murine, non-neuronal tissues. mCAT1, originally identified as a receptor for an ecotropic retrovirus(II - 23), was later found to encode a system y$^+$ activity by expression studies in *Xenopus* oocytes(II - 24,25). mCAT1 is widely expressed in various tissues and cell lines and believed to be a ubiquitous form of the CAT gene family. mCAT2 gives rise to two alternative splice forms, mCAT2a and b. mCAT2b(II - 26,27), originally identified as a T-cell early activated gene, was also found to encode a system y$^+$ activity with properties similar to that encoded by mCAT1. mCAT2b has been shown to be expressed in some types of cells including T cells and vascular smooth muscle cells. A comparative study on the distribution of mCAT2b, however, is lacking and it is not known whether neuronal/glial cells express this transporter. mCAT2a does not encode system y+ but a low affinity, high capacity transporter. The expression of mCAT2a is restricted to liver. Therefore, the L-arginine incorporation activity on B103 cells mainly due to rCAT3.

dbcAMP induced neurite outgrowth on B103 required for L-arginine - B103 line was firstly established from rat brain and its profile was corresponded to neuronal cell. Rat neuronal B103 line was known as neurite sprouting stimulated by NGF or dbcAMP same as PC12 cells. In cultures of PC12 cells, NO donors also enhanced the neuritogenic effects of NGF. The proportion of PC12 cells with neurites 48 hr after exposure to NO donors sodium nitrite (100 µM-10 mM) or sodium nitroprusside (100 nM-1 µM) plus 2.5S nerve growth factor (NGF) was approximately twice the proportion of cells with neurites in

sister cultures grown in NGF alone. Neither of the NO donors elicited neurites from the PC12 cells in the absence of NGF. The regulation of NOS activity, NO synthesis is also regulated by availability of the substrate L-arginine and that L-arginine is provided to NOS via transport from the extracellular space rather than from the intracellular metabolic pool. To clarify a physiological role of rCAT3 on B103 cell line, I tested the viability and dbcAMP induced neurite outgrowth activity on B103 cells using L-arginine lacking medium (Fig. II - 5).
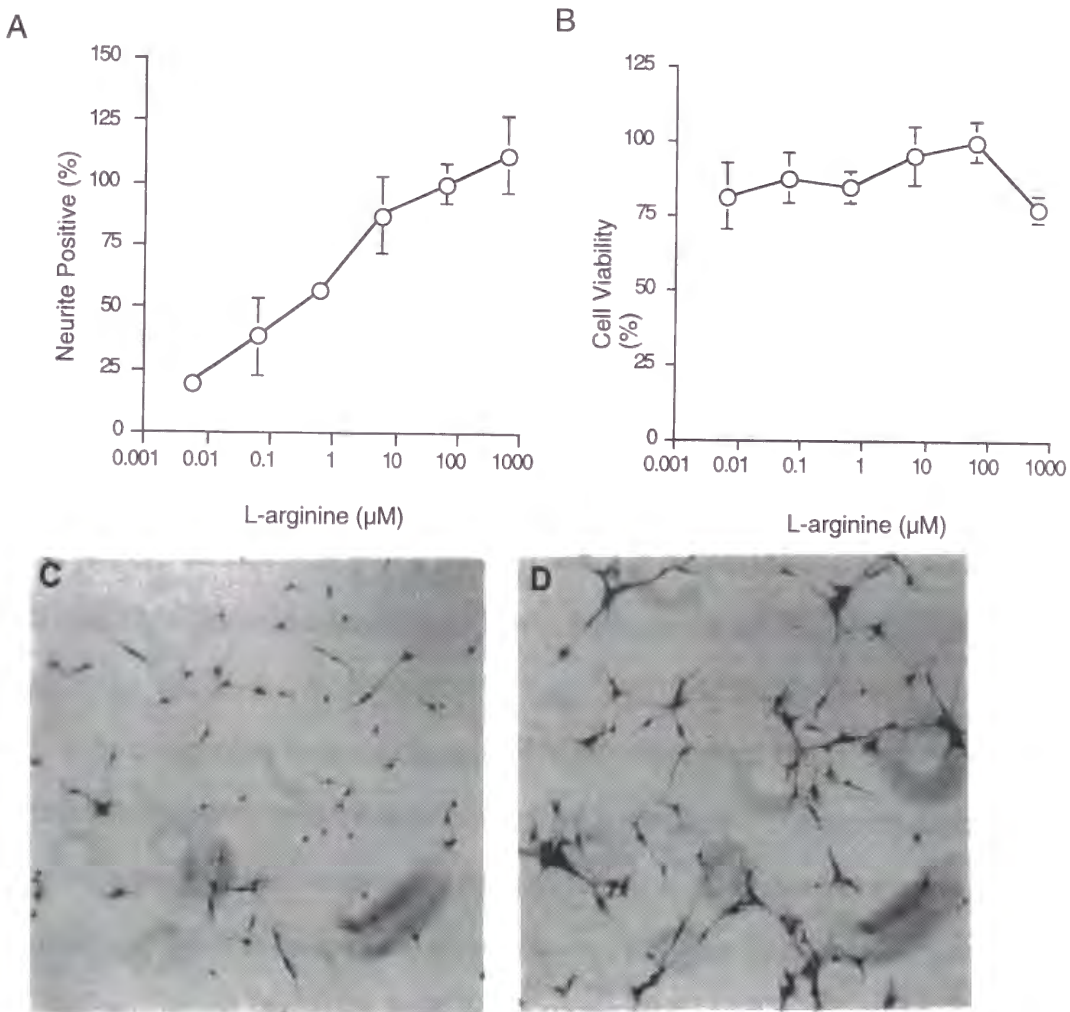
Fig. II - 5   Neurite sprouting but not cell proliferating activity of L-arginine on B103
B103 cells were seeded incubated in medium containing cAMP with various concentration of L-arginine for 48 hrs., fixed, and stained in materials and methods. total cell number and neurite positive cell number were counted. (A) The percentage of neurite positive cells, which was determined in parallel. (B) The percent increase of B103 cell numbers after 48 hrs. culture in the arginine free medium, which was determined in parallel in each experiment. Each point represents means ± SEM of triplicate determinations obtained in a single experiment. These experiments were replicated at least three times with similar results. (C, D) Typical L-arginine dependent dbcAMP induced neurite sprouting on B103. B103 cells are cultured in MEM without L-arginine(C) or with 10 μM L-arginine(D) containing 100μM dbcAMP for 48 hrs., fixed, and stained by CBB.

In L-arginine lacking medium, neurite sprouting activity on B103 cell line by dbcAMP depends upon extracellular L-arginine concentration (Fig. II - 5A). The number of neurite positive B103, but not the average length of neurite (data not shown) was increasement with extracellular L-arginine concentration. In contrast, cell number of B103 was not affected by extracellular L-arginine concentration (Fig. II - 5B). In over 0.1 mM L-arginine containing medium, the percentage of neurite positive cell was no longer increasement. Neurite sprouting is one of the character on differentiated neuronal cells. Neuronal differentiation of PC12 cells by NGF treatment was well characterized previously by several changes including growth arrest, activation of specific genes, and neurite sprouting(II - 28). High concentration of cAMP, such as 10 - 100 μM forskolin treatment, was induced neurite sprouting on PC12 cells and B103 cells like as NGF. Under these conditions, cAMP signal transduction system was activated on PC12 cells or B103 cells. Previous studies showed that neurite sprouting was required the activation of MAP kinase cascade in PC12 cells (II - 28). The dbcAMP treatment may activate MAP kinase cascade on B103 cells same as PC12 cells or neuronal cells.

In L-arginine free medium, dbcAMP stimulated B103 cells failed to neurite outgrowth, and inhibitors of rCAT3 mediated L-arginine incorporation or antisense oligonucleotide corresponded to rCAT3 did not permit neurite sprouting on B103 cells. Our observation indicates that dbcAMP induced neurite sprouting on B103 cells were required rCAT3 mediated L-arginine incorporation. Although L-arginine is one of the non - essential amino acids, B103 cells did not die. Our findings suggest that incorporated L-arginine was required some signal transduction for neurite sprouting. L-arginine is substrate of NO, so incorporated L-arginine may exchange to NO by activated

NOS in B103. In fact, in endothelial cells or neuronal cells, NO generation was dependent on extracellular L-arginine(II - 14,15). In literature, neurite sprouting on NGF treated PC12 cells were inhibited by inhibitors of NOS, L-NAME or L-NMMA(II - 13). And EGF in combination with NO generator, SIN-1, SNAP, or SNP elicited neurite sprouting(II - 29). These previous studies showed that neurite sprouting on PC12 cells required NO generation. These previous studies support our hypothesis that neurite sprouting on dbcAMP induced B103 were required L-arginine incorporation by NO generation.

Some actions of NO are mediated directly by protein modification (e.g., nitrosylation) and others by activation of soluble guanylate cyclase (soluble GC), which increases intracellular levels of guanosine 3',5'-cyclic monophosphate (cGMP)(II - 11). The NO donors stimulated a prompt increase in intracellular cGMP in PC12 cells(II - 13). Moreover their action was mimicked by addition of the membrane-permeate cGMP analogs 8-Bromo-cGMP (8-Br-cGMP) and para (chlorophenylthio)-cGMP (pCPT-cGMP) to the culture medium and by atrial natriuretic factor which stimulates particulate guanylyl cyclase(II - 13).

The dbcAMP induced neurite sprouting on B103 cells were inhibited by L-arginine depletion, but recovered by 8-Br-cGMP treatment on dose dependent manner. This data indicated that cGMP dependent mechanism exists under the downstream of L-arginine dependent signal transduction system. Our finding suggested that incorporated L-arginine dependent NO generation were activated soluble guanylate cyclase and production of cGMP.

cGMP treatment was rescued neurite outgrowth on B103 in L-arginine depletion medium - Previous studies showed that NO synthesis is also regulated by availability of the substrate L-arginine and that L-arginine is provided to NOS via transport from the extracellular space rather than from the

intracellular metabolic pool. Some actions of NO are mediated directly by protein modification (e.g., nitrosylation) and others by activation of soluble guanylyl cyclase (soluble GC), which increases intracellular levels of guanosine 3',5'-cyclic monophosphate (cGMP). Therefore, we hypothesized that incorporated L-arginine was converted to NO by NOS and increasement cellular cGMP level in B103 cells. In L-arginine depletion medium, neurite sprouting was failed on B103 cells, but application of 8-Br-cGMP may rescue. To test this hypothesis, 8-Br-cGMP treatment on B103 in L-arginine free medium. The percentage of neurite positive cells were increased with cGMP concentration in dose dependent manner (Fig. II - 6).
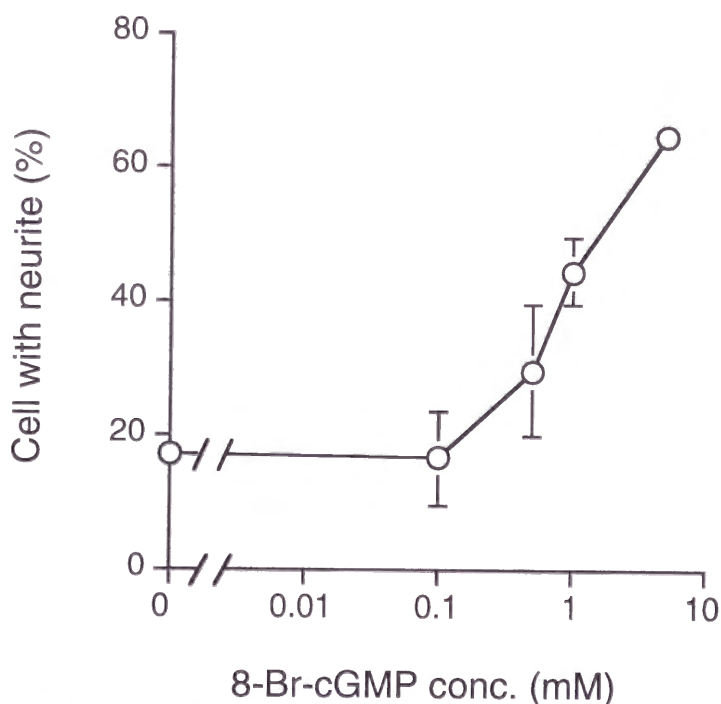
Fig. II - 6   Effects of 8-Br-cGMP on dbcAMP induced neurite sprouting in L-arginine free medium B103 cells were incubated in L-arginine free MEM containing 100 μM dbcAMP with increasing concentrations of 8-Br-cGMP , fixed, and stained by CBB. Each point represents means ± SEM of triplicate determinations obtained in a single experiment. These experiments were replicated at least three times with similar results. These experiments were replicated three times with similar results. * ; p < 0.05, significantly different from control (non paired Student's *t* test).

Neurite outgrowth activity on B103 depends upon rCAT3 mediated L-arginine incorporation - Extracellular amino acid was incorporated by the several types of amino acid transport systems distinguished by substrate specificity, cationic

amino acids such as L-arginine, L-lysine and L-ornithine share the same transporter with a sodium-independent activity, called system y^+. The presence of a high affinity system y^+ activity in B103 cells has been demonstrated by functional studies (Fig. II - 5). Therefore, to determine L-arginine transport system, required neurite sprouting activity on B103, was system y^+ or not, displacement studies was occurred. Excess L-lysine or L-ornithine treatment inhibits dbcAMP induced neurite sprouting on B103 cells at low concentration of L-arginine (10 μM) (Fig. II - 7A). But, at usual concentration of L-arginine (1 mM), L-lysine or L-ornithine treatment can not inhibit neurite sprouting on B103 (data not shown).
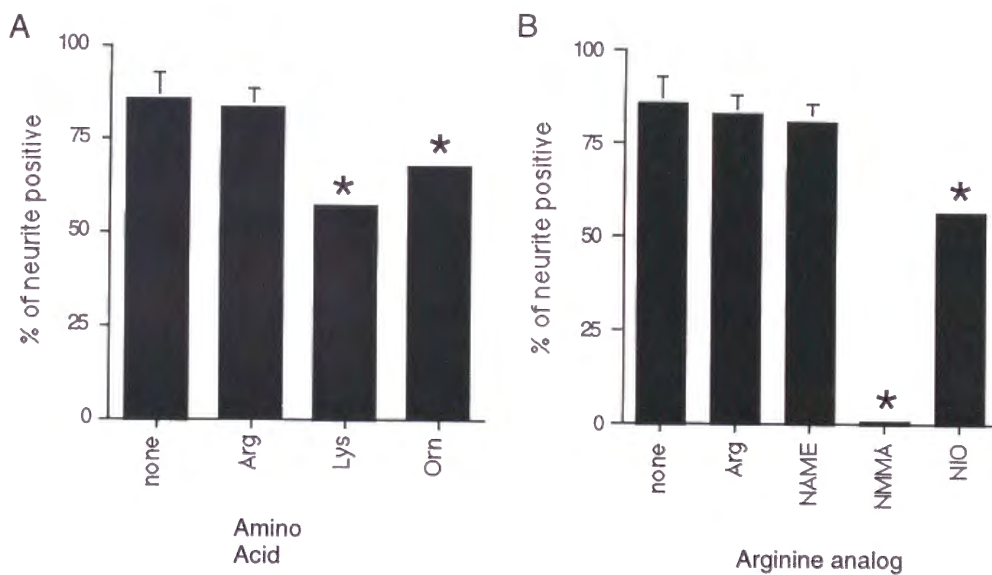
Fig. II - 7   Effects of amino acids and arginine analogs on cAMP induced neurite sprouting B103 cells were seeded incubated in medium containing cAMP, 10 μM of L-arginine , and 1 mM of indicated amino acid for 48 hrs., fixed, and stained. (A) Effects of amino acids which inhibits rCAT3 mediated L-arginine transport. None: control; Arg: L-arginine; Lys: L-Lysine; Orn: L-ornithine. (B) Effects of amino acids which inhibits nitric oxide production by nitric oxide synthase. none; control; Arg: L-arginine; NAME; L-NAME; NMMA: L-NMMA; NIO: L-NIO. The total cell number and neurite positive cell number were counted. The percent increase of neurite positive B103 cell numbers. Each point represents means ± SEM of triplicate determinations obtained in a single experiment. These experiments were replicated at least three times with similar results. These experiments were replicated three times with similar results. * ; $p < 0.05$, significantly different from control (non paired Student's t test).

Currently available NOS inhibitors are structural analogs of L-arginine and some of them also inhibit L-arginine transport in B103 cell. Therefore, I

examined the ability of three drugs, L-NMMA, L-NIO and L-NAME to inhibit dbcAMP induced neurite outgrowth in B103 (Fig. II - 7B). When B103 were exposed to 100 µM dbcAMP with 100 µM L-arginine in the presence or absence of the NOS inhibitors (1 mM), both L-NMMA and L-NIO caused a significant inhibition of the percentage of neurite positive cells, while L-NAME caused only a marginal effect. There was also a statistically significant difference between the effects of L-NMMA and L-NIO. Thus, the rank order of potency of the three drugs was L-NMMA > L-NIO >> L-NAME. Similar rank order of potency was observed for these drugs to inhibit system y+ L-arginine transport in neuronal cells.

The good agreement of pharmacological profile among previously described the profile of rCAT3, L-arginine transport activity on B103, and neurite sprouting activity on B103 lead us to the idea that When neurite sprouts stimulated by dbcAMP in B103 required L-arginine incorporation by rCAT3. Therefore, antisense oligonucleotide corresponded to rCAT3 treatment may inhibits dbcAMP induced neurite sprouting in B103.
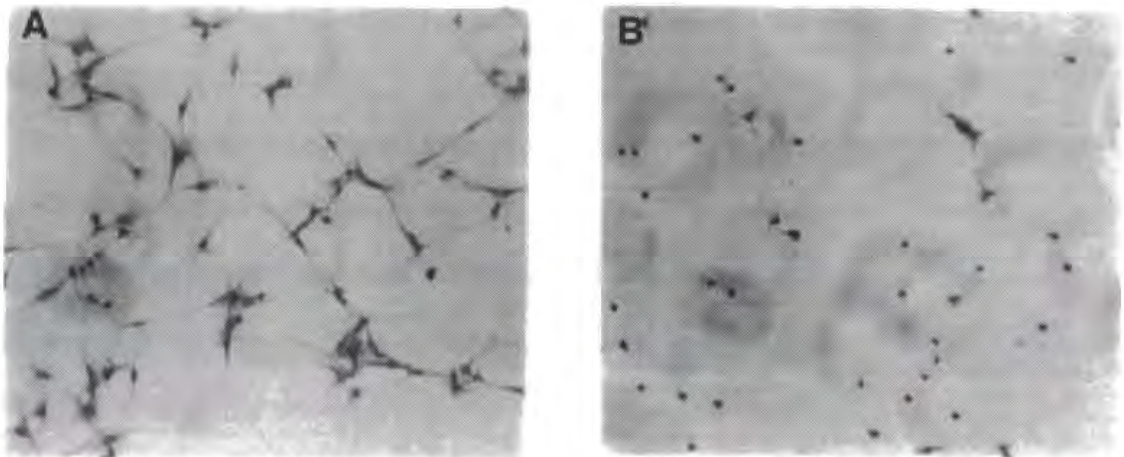

Fig. II - 8   Effects of antisense oligonucleotide of rCAT3 on cAMP induced neurite sprouting
B103 cells were incubated in MEM containing 100 µM dbcAMP, 10 µM of L-arginine , and 1 mM of sense (A) or antisense (B) oligonucleotide for 48 hrs., fixed, and stained by CBB.

The antisense oligonucleotide treatment can be reduced the level of target molecules. When B103 stimulated by dbcAMP with 100 µM L-arginine, sense (Fig. II - 8A) or antisense (Fig. II - 8B) oligonucleotide corresponded to rCAT3 was treated. Antisense oligonucleotide treatment showed significantly reduced neurite sprouting (Fig. II - 8B), in contrast, sense oligonucleotide treatment did not (Fig. II - 8A).

This paper reported that rCAT3 mediated L-arginine incorporation was required neurite sprouting on B103 neuronal cell line. Previously, I described characterization of rCAT3 in COS7 expression system, Incorporation of L-arginine mediated by rCAT3 was depended upon KCl induced depolarization(30). In neuronal cells, the depolarization was occurred by excitatory neurotransmitters such as glutamic acid. So, when neuron were excited, neurite sprouting may inhibit as a result of rCAT3 mediated L-arginine incorporation was inhibited. Previous studies showed that NOS inhibitors interfere normal retinogeniculate projection in ferret,. Thus, rCAT3 may regulate neural development by local NO control.

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CONCLUDING REMARKS

In this study, I discussed cDNA sequence, deduced amino acid sequence, activity, mRNA expression and physiological role(s) of newly cloned rCAT3, the neuron specific cationic amino acid transporter.

In part I, I found the cDNA encoding neuronal specific cationic amino acid transporter termed as rCAT3. I showed that the deduced amino acid sequence of rCAT3 has fourteen transmembrane domains and was a member of cationic amino acid transporter family. In expression study, I showed the activity of rCAT3 was a high affinity system y+ cationic amino acid transporter, and rCAT3 dependent L-arginine incorporation was specific only inhibited by various L-arginine analogs except L-NAME, and dependent upon KCl induced depolarization nernst equation dependent manner.

In part II, I described that rCAT3 controls neurite sprouting via NO - cGMP cascade. The expression of rCAT3 mRNA was restricted to brain determined with northern blot analysis, and in almost neuronal cells in *in situ* hybridization analysis. The neurite sprouting stimulated by cAMP on B103, derived from rat brain neuron, showing high expression of rCAT3, was dependent on extracellular L-arginine. Its sprouting was completely inhibited by treatment of antisense oligonucleotide against rCAT3.

In conclusion, rCAT3 was neuron specific high affinity system y+ cationic amino acid transporter, and it controls neurite sprouting via NO - cGMP pathway.

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